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Award Number: W81XWH-05-1-0045

TITLE: MT 2A Phosphorylation by PKC Mu/PKD Influences Chemosensitivity to
Cisplatin in Prostate Cancer

PRINCIPAL INVESTIGATOR: Balaji C. Kethandapatti, M.D., FRCS
Meena Jaagi
David J. Smith
Wenguang Zhang
Cheng Du
Lynette M. Smith
Anton Galich

CONTRACTING ORGANIZATION: University of Nebraska Medical Center
Omaha, NE 68198-5100

REPORT DATE: November 2005

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE (DD-MM-YYYY) 01-11-2005		2. REPORT TYPE Annual		3. DATES COVERED (From - To) 1 Nov 2004 – 31 Oct 2005	
4. TITLE AND SUBTITLE MT 2A Phosphorylation by PKC Mu/PKD Influences Chemosensitivity to Cisplatin in Prostate Cancer				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-05-1-0045	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Balaji C. Kethandapatti, M.D., FRCS, Meena Jaagi, David J. Smith, Wenguang Zhang, Cheng Du, Lynette M. Smith, Anton Galich E-mail: kcbalaji@surgery.unmc.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Nebraska Medical Center Omaha, NE 68198-5100				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT The metallothioneins (MT) are a family of small molecular weight trace metal and free radical scavenging proteins well established to play a role in resistance to chemotherapy and radiation in human cancer. MT gene expression is up regulated in response to the presence of heavy metal ions such as zinc. The activation of MT gene expression in response to zinc treatment in LNCaP and C4-2 prostate cancer (PC) cells was shown by western blotting and DNA microarray analysis. Chemotherapy and radiation sensitivity assays of cells following treatment with cisplatin or radiation were performed in the presence, or absence, of 150µM ZnSO4 and cell viability measured after 72 hours by MTS viability, clonogenic and flow cytometry assays. Increasing concentrations of ZnSO4 up regulated MT expression in a dose dependent manner. Microarray analysis demonstrated specific increase in MT expression. Cells treated with zinc demonstrated a significantly decreased sensitivity to cisplatin compared to controls (p < 0.05). We have established a physiological in vitro cell line model of MT induction using Zn, which is significantly associated with resistance to cisplatin chemotherapy in PC. Immunohistochemistry (IHC) analysis for MT expression in human prostate cancer specimens confirmed nuclear and cytoplasmic expression of MT in majority of specimens. However, there was no significant difference in expression between various grades of PC.					
15. SUBJECT TERMS Advanced prostate cancer, Chemoresistance, Metallothionein 2A, MT 2A Protein kinase C Mu, PKC Mu					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 30	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

Table of Contents

Title: MT 2A Phosphorylation by PKCMu/PKD Influences Chemosensitivity to Cisplatin in Prostate Cancer.

Cover.....	1
SF 298.....	2
Introduction.....	4
Body.....	4-9
Key Research Accomplishments.....	10
Reportable Outcomes.....	10
Conclusions.....	10
References.....	10
Appendices.....	10

Introduction

Our preliminary studies had demonstrated that kinase domain of Protein kinase D1, a novel serine threonine kinase, interacts with Metallothionein 2A (MT 2A), which belongs to the family of small molecular weight proteins called metallothioneins (MT) that scavenge trace metals and free radicals and are associated with resistance to chemotherapy and radiation in human cancers¹. Our studies further demonstrated the C4-2 prostate cancer cells that express higher levels of MT compared to its parental LNCaP cells² are selectively more resistant to trace metal containing chemotherapy agent cisplatin compared to LNCaP cells. Our *in vitro* experiments also demonstrated that MT 2A was phosphorylated by PKD1. Therefore we hypothesized “*Alteration in MT 2A expression influences chemoresistance to cisplatin in prostate cancer. PKC Mu/PKD kinase activity influences sensitivity to cisplatin by MT 2A phosphorylation in prostate cancer. The expression of MT 2A is quantitatively increased in progressive human prostate cancer*”.

We proposed to establish the stated hypothesis through 3 aims.

Aim 1. To determine that alteration in MT 2A expression influences resistance to cisplatin in prostate cancer.

Aim 2. Inhibition of PKC Mu/PKD kinase activity and its influences on chemoresistance in prostate cancer cells by modulating the phosphorylation of MT 2A.

Aim 3. To quantify and qualitatively evaluate MT 2A protein expression in progressive human prostate cancer.

Body

Task 1: *To determine that alteration in MT 2A expression influences resistance to cisplatin in prostate cancer.*

1a: Effect of MT 2A over expression influences resistance to cisplatin in prostate cancer

Experiment 1: Cloning and Expression of MT 2A protein in LNCaP prostate cancer and MT 2A null CHO cells. Using standard laboratory cloning techniques we have clone the MT 2A cDNA sequence with His tag into the expression plasmid pcDNA3.1 vector. We have added the His tag to MT 2A cDNA sequence in order to differentiate MT 2A with other MT isoforms, as commercially available E-9 antibody recognizes most of the MT isoforms. LNCaP and CHO cells, obtained from ATCC, were grown in DMEM and Hams F12 medium respectively supplemented with 10% Fetal Bovine Serum (Invitrogen), 100 units of penicillin/ml, 100µg of streptomycin/ml at 37°C in humidified 5% CO₂ and 95% air. Stable transfections were performed by lipofection with Lipofectamine reagent (Invitrogen Inc.,) in 6 well plates according to the manufacturer's protocol. We are in process of selecting MT2A positive stable clones.

While we are in the process of creating stable MT 2A transfectants, we explored alternative and physiologically relevant methods of over expressing MTs. Compared to all other organs in human body, prostate has the highest concentration of Zn (150 μg compared to 20-50 μg per gram of tissue). Because MT is responsive to trace metals including Zn, we proceeded to establish an *in vitro* prostate cancer cell line model system to study the of MT induction by Zn on cellular phenotype including resistance to cisplatin chemotherapy and radiotherapy.

INDUCTION OF METALLOTHIONEIN EXPRESSION BY ZINC SULFATE

Firstly, we established that prostate cancer cell tolerate very high concentrations of Zn. As shown in Figure 1A, while the concentration of ZnSO_4 up to 200 μM did not significantly affect the viability of prostate cancer cells, a majority of control ovarian cancer CP70 cells died at concentrations above 50 μM . These results demonstrate the characteristic tolerance of C4-2 PC cells to high concentrations of zinc. Secondly, treatment of LNCaP and C4-2 cells with increasing concentrations of ZnSO_4 demonstrates up regulation of MT gene expression (Fig. 1B). The expression of Protein kinase D1, studied as control, is unaltered by the ZnSO_4 . The addition of 150 μM MgSO_4 to LNCaP and C4-2 cells did not affect the expression of MT, which confirms the specificity of induction of MT by Zn (Fig. 1C). Furthermore, we established the specificity of MT induction in this cell line model by carrying out microarray analysis (Figs 1D and 1E) and validated the microarray results on MT expression by conventional Western blotting (Figs 1B and 1C).

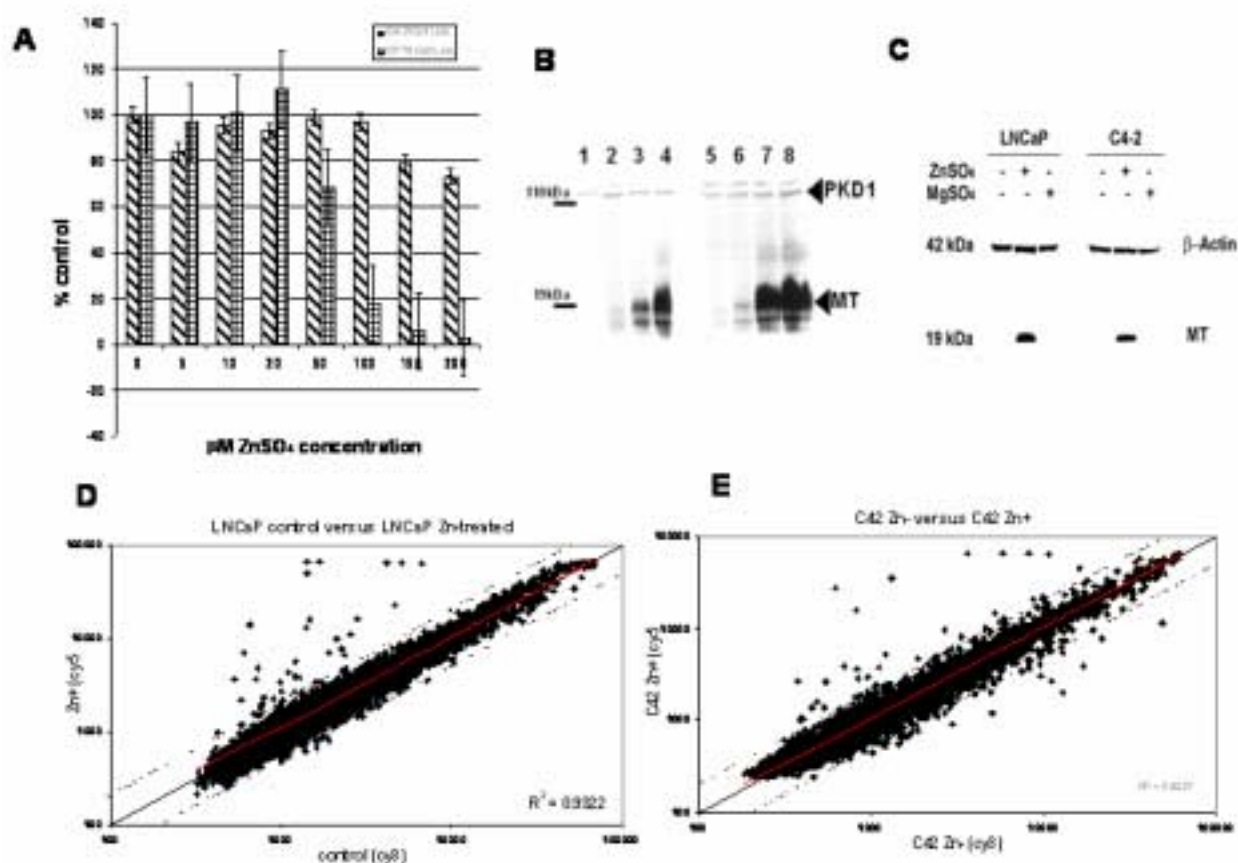
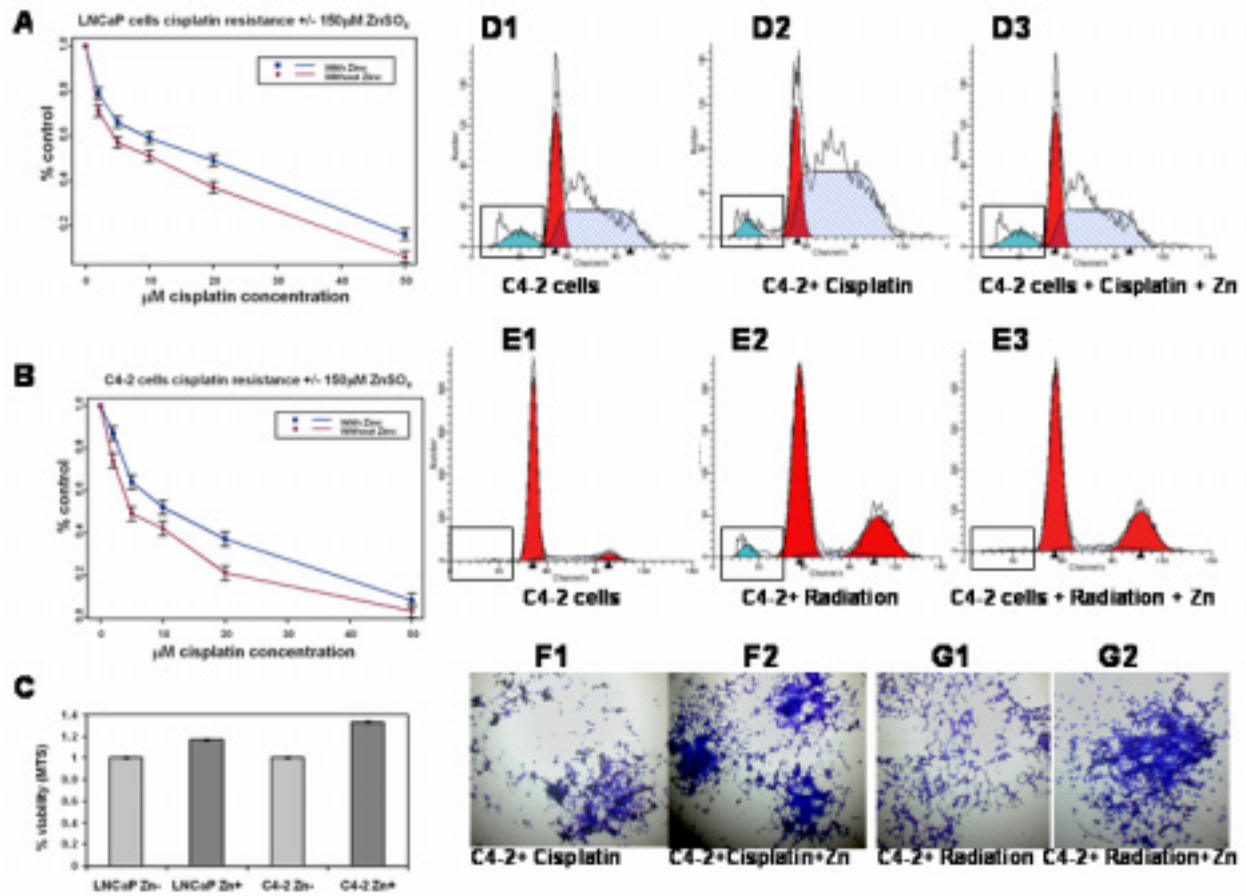


Fig. 1A: C4-2 PC cells characteristically tolerate treatment with high concentrations of ZnSO_4 . C4-2 PC cells and CP 70 ovarian cancer cells (control) were grown to 80% in the presence of

ZnSO₄. Cells were then incubated at 37°C for 72 hours. Cell growth and viability was measured by MTS assay using the aqueous non-radioactive cell proliferation assay kit (Promega, Madison, WI). Whereas the C4-2 PC cells did not demonstrate significant decrease in viability in spite of high concentration of ZnSO₄ up to 200 µM, a majority of CP 70 ovarian cancer cells died at concentrations above 50 µM. Figs. 1B and 1C: Metallothionein expression is up regulated by ZnSO₄, but not by MgSO₄. LNCaP and C4-2 cells were plated out in 6 well plates 24 hours prior to the addition of increasing concentrations of ZnSO₄, or MgSO₄. After 72 hours incubation the cells were lysed and analyzed by western blotting as described in method section. In fig 1B, lanes 1-4 show LNCaP cells, lanes 5-8 show C4-2 cells, treated with 0, 50, 100 and 150µM ZnSO₄. The positions of the protein markers are shown on the left. Protein kinase D1 is visible as a band in all lanes of 120kDa in size and its expression is not affected by zinc treatment, whereas, MT expression is clearly increased in a dose responsive manner. Induction of metallothionein expression in response to increasing ZnSO₄ concentration is shown by the increase in size of a band at around 10kDa. Fig 1C shown that MT expression is clearly increased in LNCaP cells treated with ZnSO₄, in LNCaP and C4-2 cells, but is no changed in LNCaP cells treated with MgSO₄. 1D and 1E: Alterations in gene expression in LNCaP and C4-2 cells by 150µM Zinc Sulfate. Scatter plots showing the gene expression ratio changes in the cell lines LNCaP (D) and C4-2 (E) in the presence or absence of 150µM Zinc Sulfate. Dots above the upper dashed line indicate genes expressed significantly (>2 logs) higher in cells treated with Zn. The MT isoforms are visible as the most highly up regulated genes at the top of each plot. Dots within in the dashed lines represent genes whose expression does not change significantly (<2 logs). Dots below the lower dashed line indicate genes expressed significantly (>2 logs) lower in cells treated with zinc. R² values indicate the linear regression of the data points.

OVEREXPRESSION OF MT BY ZN INDUCES CISPLATIN RESISTANCE

We incubated LNCaP and C4-2 cells for 72hours in the presence or absence of 150µM ZnSO₄ and increasing concentrations of cisplatin. The IC₅₀ for cisplatin induced cell death was 10 µM in cells not treated with ZnSO₄, which was therefore selected as study concentration for clonogenic and apoptosis assays. The results are shown in Figs. 2A and 2B. The cells treated with ZnSO₄ were significantly (p<0.005) more resistant to cisplatin at all study concentrations, strongly suggesting an association between MT induction and resistance to cisplatin in PC. The flow cytometric analyses were carried out and the apoptotic cell populations in sub-G1 phase were quantified. A significant decrease in cell death occurred in LNCaP and C4-2 cells treated with cisplatin; LNCaP: 10%, LNCaP + cisplatin: 30.64%, LNCaP +cisplatin +Zn: 16.64%; C4-2: 8.73%, C4-2+ cisplatin: 14.24%, C4-2+cisplatin +Zn: 9.12%; p value < 0.001 (Fig. 2 D1-D3) or radiation LNCaP: 1.05%, LNCaP + radiation: 9.26%, LNCaP + radiation + Zn: 1.17%, C4-2: 0.34%, C4-2+ radiation: 6.35%, C4-2+ radiation+Zn: 1.13%; p value < 0.001 (Fig. 2E1-E3) following pretreatment with Zn compared to untreated cells;. There were no changes in cell cycle phases. In an effort to establish that inhibition of apoptosis by ZnSO₄ in PC cells treated with cisplatin or radiation persists to influence cell growth, clonogenic assays were performed. The number of colonies were significantly increased in LNCaP and C4-2 cells treated with Zn and cisplatin (median 104.5, range 102-108 and median 38.5, range 30-45; p value = 0.03, Fig. 2F1-F2) and by 2-fold in Zn and radiation group compared to cisplatin or radiation treated cells without Zn (LNCaP cells; 99, 56; C4-2 cells; 98, 48, Fig. 2G1-G2).



Figs. 2A and 2B: Cisplatin dose response experiments. Experiments carried out at cisplatin doses ranging from 0 through 50µM, with cell viability measured at 3 days. LNCaP (Fig. 2A) and C4-2 (Fig. 2B) cells. Each experiment was carried out in triplicate. Cell growth and viability were measured by MTS assay, absorbance readings were taken at wavelengths of 490 and 655nm and the average absorbance for each cell line and concentration of drug was calculated. Error bars indicate mean standard error. The data points for zinc treated cells are shown as squares (■) with untreated cells shown as diamonds (◆). 2C: Radiation sensitivity of LNCaP and C4-2 cells in the presence of 150µM ZnSO₄. LNCaP and C4-2 cells were grown to 80% confluence, plated in 96-well plates and incubated overnight in the presence or absence of 150µM ZnSO₄ as described in the previous experiment. Cells were then exposed to 5Gy of γ-radiation, grown for a further 72 hours and cell growth and viability was measured by MTS assay. The experiments were performed in triplicate and means compared. Results are shown as a bar graph comparing cells exposed to radiation in the presence or absence of 150µM ZnSO₄. Error bars show standard error. 2D-E: Analysis of apoptosis by flow cytometry: C4-2 cells treated with 10µM cisplatin or 5Gy radiation in presence or absence of 150µM of ZnSO₄ for 48 hours were analyzed. The figure demonstrates a significant decrease in apoptosis (sub G0 peak shown in box) in cells treated with ZnSO₄ compared to untreated cells; C4-2 cells only 8.73%, C4-2 cells + cisplatin: 14.24%, C4-2 cells + cisplatin + Zn: 9.12%; p value < 0.001(D1-D3), C4-2 cells: 0.34%, C4-2 cells + radiation: 6.35%, C4-2 cells + radiation + Zn: 1.13%; p value < 0.001(E1-E3). 2F-G: A representative view of clonogenic assay demonstrating increased number of colonies in C4-2 cells treated with 10µM cisplatin (F1-2) or 5Gy radiation (G1-2) and 150µM of ZnSO₄ compared to cells not treated with ZnSO₄.

1b: Down regulation of MT2A by RNAi leads to increased sensitivity to cisplatin.

We incubated C4-2 cells for 24 hours in the presence of RPMI media. RPMI media was exchanged for OPTI-MEM media at 24 hours and 150 μ M ZnSO₄ was added to the appropriate cells. Cell transfection with control siRNA and MT siRNA was performed at 48 hours using a DNA-Lipofectamine 200 kit. Cells were further incubated for 48 hours. We are currently in the process of confirming down regulation of MT by Western blot.

Methods: Gene silencing by short interfering double stranded RNA (siRNA), determination of chemo resistance by MTS assay, In vitro kinase assays.

Timeframe: 6-24 months

Deliverable: Analyze MT 2A phosphorylation and in PKC Mu silenced cells.

Task 2: Inhibition of PKC Mu/PKD kinase activity and its influences chemoresistance in prostate cancer cells by modulating the phosphorylation of MT 2A.

Methods: Determination of MT 2A phosphorylation using phosphoserine specific antibodies and kinase assays in cells treated with PKC inhibitors, determination of chemo resistance by MTS assay

Timeframe: 18-36 months

Deliverable: To demonstrate that inhibition of PKC Mu/PKD kinase activity influences chemoresistance in prostate cancer cells by modulating the phosphorylation of MT 2A

We plan to work on this task in the next 24 months.

Task 3: To quantify and qualitatively evaluate MT 2A protein expression in progressive human prostate cancer.

Methods: Search patient records, identify cohort of about 20 patients for study, obtain tissue samples, stain and process tissue. QFIA and IHC analysis of slides, analysis of data.

Timeframe: 6-36 months

Deliverable: Demonstrate MT 2A alteration in human prostate cancer tissue.

IMMUNOHISTOCHEMISTRY (IHC) ANALYSIS OF MT EXRESSION IN PROSTATE CANCER:

We carried out immunohistochemistry (IHC) studies on a total of 33 human paraffinized prostate cancer tissue specimens. Adequate grading information was available in 31 of these specimens, of which 20 and 11 specimens consisted of Gleason 5-6 and Gleason 7-10 respectively. MT expression was demonstrable in both nucleus and cytoplasm of prostate cancer cells. However, there was no significant difference in expression of MT between various grades of prostate cancer, pathological stage or serum preoperative prostate specific antigen (PSA) levels (Table 1).

	MT staining		
	absent (1-) (n=10)	present (n=23)	P-value
Gleason 5-6 7-10	6 (60%) 4 (40%)	14 (67%) 7 (33%)	1.00
Stage 2 3-4	8 (80%) 2 (20%)	19 (83%) 4 (17%)	1.00
PSA 0 - 4 > 4	- 9 (100%)	3 (17%) 15 (83%)	0.53

Because we were unable to demonstrate difference in MT expression by IHC studies, we have started to explore MT protein expression by Quantitative Fluorescence Image Analysis (QFIA), which is more sensitive than IHC in detecting differences in protein expression.

QUANTITATIVE FLUORESCENCE IMAGE ANALYSIS OF MT EXRESSION IN PROSTATE CANCER:

QFIA represents a sensitive and reproducible technique for quantifying protein expression.

During this first year of funding, we have established the optimum antibody titers and incubation times for analyzing MT expression in LNCaP prostate cancer cells. A concentration of 50,000 LNCaP cells were harvested, fixed and stored at -80°C. One to three days prior to fluorescence labeling, cryopreserved suspensions were thawed, captured by filtration units, cells blotted and fixed onto slides. Slides are then labeled using a BioGenex autostainer first with the primary antibody (Anti-MT) followed by secondary antibody (goat anti-mouse IgG coupled with Alexa Fluor 488 Molecular Probes). We have determined the optimal primary (1/200, Anti-MT) and secondary antibody (1/100, Molecular Probe) concentrations for detecting metallothionein expression in LNCaP cells. We have also determined optimal antibody incubation times for each (1 hour for each antibody). Negative controls were treated with mouse IgG Isotype control rather than primary antibody. Expression was quantified using the mean pixel intensity of the fluorescence signal following image capture (Fig 3).

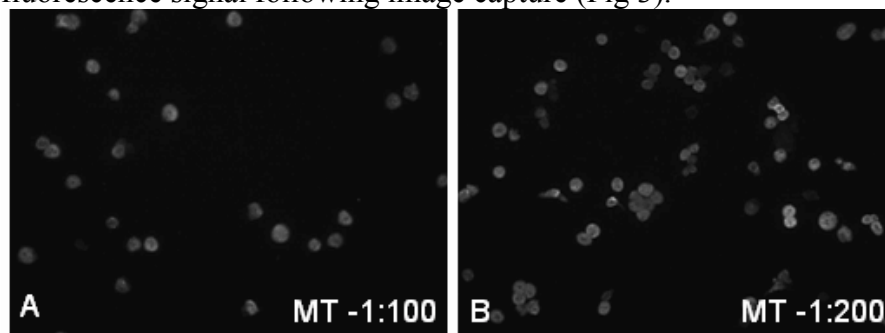


Figure 3; Quantifiable fluorescent signals following labeling of fixed LNCaP cells with MT antibody at concentrations of 1:100 or 1:200. A concentration of 1:200 produces better signal intensity

Key Research Accomplishments

We have confirmed that MT induction by Zn increases resistance to cisplatin in prostate cancer cells. In the process of establishing this result, we have developed an excellent physiologically relevant prostate cancer cell line model system to study the effects of MT expression by Zn. We were unable to demonstrate significant difference in cytoplasmic or nuclear expression of MT between various grades of prostate cancer, suggesting that MT expression may not be associated with prostate cancer progression.

Reportable Outcomes

1. David J Smith, Meena Jaggi, Prema Rao, Anton Galich, Wenguang Zhang and **K.C. Balaji**; Expression of Metallothioneins is Associated with Resistance to Cisplatin and Radiation in Prostate Cancer Cells (Manuscript submitted for publication, Urology, November 2005, Appendix 1)
2. David J. Smith, Meena Jaggi, Lynette M. Smith, **K.C. Balaji**: Expression of Metallothioneins is Associated with Resistance to Both Cisplatin Chemotherapy and Radiation in Prostate Cancer Cells, SCS, AUA, 84th Annual Meeting, Austin, Texas, Abstract #49, 9/05
3. David J Smith, Meena Jaggi, Prema S Rao, Lynette M Smith, **K.C. Balaji**, Expression of Metallothioneins is Associated with Resistance to Both Cisplatin Chemotherapy and Radiation in Prostate Cancer Cells; J Urol, Vol 173, No: 4, Abs 397, 2005

Conclusions

MT expression is strongly associated with resistance to cisplatin chemotherapy in human prostate cancer cells.

References

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2. Rao, P. S., Jaggi, M., Smith, D. J., Hemstreet, G. P. & Balaji, K. C. Metallothionein 2A interacts with the kinase domain of PKCmu in prostate cancer. *Biochem Biophys Res Commun* 310, 1032-8 (2003).

Appendices

Attached, submission to Urology, "Metallothioneins and Resistance to Cisplatin and Radiation in Prostate Cancer".

METALLOTHIONEINS AND RESISTANCE TO CISPLATIN AND RADIATION IN PROSTATE CANCER

DAVID J. SMITH^{1#}, MEENA JAGGI^{1#}, WENGUANG ZHANG¹

ANTON GALICH¹, CHENG DU¹, SAMUEL P. STERRETT¹, LYNETTE M. SMITH² AND

K.C. BALAJI^{1*}

¹Division of Urological Surgery and ²Department of Preventive and Societal Medicine

University of Nebraska Medical Center

982360 Nebraska Medical Center,

Omaha, Nebraska 68198-2360, USA

[#]These two authors contributed equally

*Corresponding author:

K.C. Balaji, M.D., FRCS

Director of Urological Oncology Research,

Professor,

Urologic Surgery, 982360,

University of Nebraska Medical Center,

Omaha, Nebraska 68198-2360

USA

Tel: 402- 559- 9041, Fax: 402- 559- 6529

E-mail: kcbalaji@unmc.edu

ACKNOWLEDGEMENTS

This work was supported by a seed grant from the University of Nebraska Foundation, Omaha, Nebraska and by a grant from the Department of Defense/PC Research grant

PC040484. We thank the Cell Analysis Facility at UNMC, which is supported by the Nebraska Research Initiative, for providing assistance with flow cytometry analysis.

ABSTRACT

Purpose; The metallothioneins (MT) are a family of small molecular weight trace metal and free radical scavenging proteins well established to play a role in resistance to chemotherapy and radiation in human cancers. MT gene expression is up regulated in response to the presence of metal ions such as zinc. Because prostate tissue has the highest concentration of zinc in the human body, in this study we analyzed the effect of MT induction by zinc in prostate cancer.

Materials and Methods; The activation of MT gene expression in response to zinc treatment in LNCaP and C4-2 prostate cancer (PC) cells was shown by western blotting and DNA microarray analysis. Chemotherapy and radiation sensitivity assays of cells following treatment with cisplatin or radiation were performed in the presence, or absence, of 150 μ M ZnSO₄ and cell viability measured after 72 hours by MTS viability, clonogenic and flow cytometry assays. Experiments were repeated 3 times and the data analyzed.

Results; Increasing concentrations of ZnSO₄ up regulated MT expression in a dose dependent manner. Microarray analysis demonstrated specific increase in MT expression. Cells treated with zinc demonstrated a significantly decreased sensitivity to cisplatin and radiation treatment compared to controls ($p < 0.05$).

Conclusions; Our data confirms that treatment of PC with Zn causes an increase in MT expression, which is significantly associated with resistance to cisplatin chemotherapy and radiation in PC. Therapeutic targeting of MT may therefore provide a means to overcome resistance to radiation and cisplatin chemotherapy in prostate cancer.

INTRODUCTION

The prostate gland is distinct from other organs in human body by its unusually high concentration of Zn (150µg/g wet weight, compared to 20-50µg/g wet weight in other organs) ¹. Among genes responsive to zinc induction, metallothioneins (MT) are an important family of proteins associated with resistance to chemotherapy and radiation in human cancers ². There are several reports demonstrating MT over expression as a useful prognostic factor for tumor progression and is implicated in causing resistance to chemotherapy in a variety of human cancers ³⁻⁵. The MT 1A, MT 1E, MT 1X, and MT 2A isoforms of MT gene have been demonstrated in normal human prostate ⁶. Because MT are trace metal responsive genes, we evaluated the effect of MT induction by Zn on resistance to radiation and cisplatin treatment in PC cells, which may provide unique opportunities to manipulate the cellular events in a prostate cell.

MATERIALS AND METHODS

WESTERN BLOTTING OF $ZnSO_4$ TREATED CELLS

The LNCaP and C4-2 PC cells were plated in 6-well plates (3×10^5 cells/well) in the presence of 0, 25, 50, 100, 150 μM $ZnSO_4$, or 150 μM $MgSO_4$ and grown for 72 hours. Cells were then lysed and Western blotting was carried out as previously described⁷ using E9 Metallothionein antibody (Dako, Carpinteria, CA), anti β -actin antibody (Sigma St Louis, MO) and horseradish peroxidase conjugated secondary antibodies (Promega, Madison, WI).

OLIGONUCLEOTIDE ARRAY GENE EXPRESSION ANALYSIS

DNA microarray experiments were carried out using 40 μg total RNA extracted from LNCaP or C4-2 cells treated with or without 150 μM $ZnSO_4$. DNA oligonucleotide microarray slides were obtained from our institutional core facility. These slides contain DNA oligomers representing 12,144 known human genes and a selection of expressed sequence tags (ESTs) of unknown genes. Hybridizations were carried out by the microarray core facility according to standard protocols and scanned with a ScanArray 4000 confocal laser system (Perkin-Elmer, Wellesley, MA). Background fluorescence was subtracted, and normalization and filtering of the data were performed using the QuantArray software package (Perkin-Elmer, Wellesley, MA). Next, expression ratios were calculated for each feature and linear regression values calculated for the data in each experiment.

CELL GROWTH AND VIABILITY ASSAY

The effect on cell growth and viability of C4-2 PC and CP70 ovarian cancer cells treated with increasing concentrations of $ZnSO_4$ or $MgSO_4$ (0 – 200 μM) were studied by MTS assay using the manufacturer's protocol (Promega, Madison, WI).

CHEMOTHERAPY AND RADIATION SENSITIVITY ASSAYS

LNCaP and C4-2 cells were plated in 96-well plates (5×10^3 cells/well) in the presence or absence of $150 \mu\text{M}$ ZnSO_4 . Cells were then incubated at 37°C for 24 hours, before the addition of cisplatin at concentrations of 0, 2, 5, 10, 20 and $50 \mu\text{M}$ and incubated for a further 72 hours. Cell growth and viability was measured by MTS assay, absorbance readings were measured at wavelengths of 490 and 655 nm and these values were then normalized to absorbance of untreated control cells. The experiments were performed in triplicate and means compared.

For radiation experiments, we used a dose of 5 Gy of γ -radiation (Gammaradiator 100), which caused significant cell mortality (30-40% in treated cells) but not complete cell death, and cell growth and viability measured by MTS after 72 hours as described above. The experiments were performed in triplicate and the means of the irradiated and non-irradiated cells treated with or without ZnSO_4 were compared following correction of zinc induced mild cytotoxicity.

CELL CYCLE AND APOPTOSIS ASSAY BY FLOW CYTOMETRY

The LNCaP and C4-2 cells were grown at a density of about 5×10^5 cells on 25 cm^2 dishes as described earlier, and treated with $10 \mu\text{M}$ cisplatin or a dose of 5 Gy of γ -radiation, in the presence or absence of $150 \mu\text{M}$ ZnSO_4 for 48 hours. The cells were trypsinized, centrifuged, washed in PBS, fixed with cold ethanol for 15 min, resuspended and stained with Telford reagent at 4°C overnight⁸. All flow cytometry measurements were done using FACS Calibur flow cytometer (BDIS San Jose, CA), and data from at least 10,000 cells were collected using ModFit LT 2.0 for cell cycle and apoptosis. Apoptosis was reported as a percentage of cells in the sub-G0 peak.

CLONOGENIC ASSAYS

LNCaP and C4-2 cells were plated in 6 well plates (3×10^5 cells/well) and treated with with 10 μ M cisplatin or a dose of 5Gy of γ -radiation (Gammaradiator 100), in presence or absence of 150 μ M ZnSO₄ for 48hours. Drug-containing media were removed, and cells were then incubated in drug-free media for 2weeks. Colonies were then fixed, stained by ProtocolTM stain (Fisher Scientific, Middletown, VA) according to manufacturer's protocol and counted.

STATISISCAL ANALYSIS

A mixed ANOVA model was used to compare the mean values between groups. Chi-square tests were used to compare the amount of apoptosis between the groups. When significance was found between groups, pairwise tests were conducted and the Bonferroni method was used to adjust p-values for multiple comparisons. The Wilcoxon rank sum test was used to compare clonogenic assay results between the cisplatin and cisplatin + Zn groups. P-values < 0.05 are considered statistically significant. SAS software was used for the analysis.

RESULTS

EFFECT OF ZN ON CELL GROWTH AND VIABILITY OF PC CELLS

As shown in Figure 1A, while the concentration of ZnSO₄ up to 200µM did not significantly affect the viability of PC cells, a majority of control ovarian cancer CP70 cells died at concentrations above 50 µM. These results demonstrate the characteristic tolerance of C4-2 PC cells to high concentrations of zinc.

INDUCTION OF METALLOTHIONEIN EXPRESSION BY ZINC SULFATE

Treatment of LNCaP and C4-2 cells with increasing concentrations of ZnSO₄ induced MT protein expression (Fig. 1B). The expression of Protein kinase D1, used as a control, is unaltered by the ZnSO₄. The addition of 150µM MgSO₄ to LNCaP and C4-2 cells did not affect the expression of MT, which confirms the specificity of induction of MT by Zn (Fig. 1C).

MICROARRAY ANALYSIS CONFIRMS MT INDUCTION IN LNCaP AND C4-2 CELLS BY ZNSO₄

The gene induction by ZnSO₄ in both LNCaP and C4-2 cells showed similar patterns (Figs. 1D and 1E). Linear regression values were above 0.9 for all data sets, indicating a high degree of linearity. Only 40/12, 144 (0.33%) and 26/12, 144 (0.21%) of genes were differentially expressed between the treated and untreated samples in LNCaP and C4-2 cells respectively. Interestingly, within the tiny fraction of the differentially expressed genes between the treated and untreated samples, MT 1 and MT 2 were strongly up regulated in both cell lines. The MT 3 fluorescence signal was low in both cell lines suggesting low cellular expression, which concurs with the data in the literature that MT3 is expressed predominantly in neuron cells⁹. While a number of genes were up regulated, only 2 genes in LNCaP and 9 genes in C4-2 were

significantly down regulated, none of which were highly expressed (Table 1A and 1B). We have established that treatment of PC with zinc is an excellent *in vitro* model to study the effects of MT induction.

TREATMENT OF PC CELLS WITH ZINC INDUCES CISPLATIN RESISTANCE

We incubated LNCaP and C4-2 cells for 72hours in the presence or absence of 150 μ M ZnSO₄ and increasing concentrations of cisplatin. The IC₅₀ for cisplatin induced cell death was 10 μ M in cells not treated with ZnSO₄, which was therefore selected as study concentration for clonogenic and apoptosis assays. The results are shown in Figs. 2A and 2B. The cells treated with ZnSO₄ were significantly ($p < 0.005$) more resistant to cisplatin at all study concentrations, strongly suggesting an association between MT induction and resistance to cisplatin in PC.

ZINC INCREASES PC CELL RESISTANCE TO GAMMA RADIATION

In addition to cisplatin resistance, MT contribute to radiation resistance by scavenging the free-radicals generated by ionizing radiation¹⁰. We explored whether induction of MT expression increased the resistance of PC cells to gamma radiation (Fig. 2C). The difference in the resistance to radiation between zinc treated and untreated cells was highly significant ($p < 0.001$), indicating that the addition of 150 μ M zinc induces a protective effect against radiation damage on both cell lines.

TREATMENT OF PC CELLS WITH ZINC DECREASES CISPLATIN OR RADIATION INDUCED APOPTOSIS

The flow cytometric analyses were carried out and the apoptotic cell populations in sub-G1 phase were quantified. A significant decrease in cell death occurred in LNCaP and C4-2 cells

treated with cisplatin; LNCaP: 10%, LNCaP + cisplatin: 30.64%, LNCaP +cisplatin +Zn: 16.64%; C4-2: 8.73%, C4-2+ cisplatin: 14.24%, C4-2+cisplatin +Zn: 9.12%; p value < 0.001 (Fig. 2 D1-D3) or radiation LNCaP: 1.05%, LNCaP + radiation: 9.26%, LNCaP + radiation + Zn: 1.17%, C4-2: 0.34%, C4-2+radiation: 6.35%,C4-2+radiation+Zn: 1.13%; p value < 0.001 (Fig. 2E1-E3) following pretreatment with Zn compared to untreated cells;. There were no changes in cell cycle phases. In an effort to establish that inhibition of apoptosis by ZnSO₄ in PC cells treated with cisplatin or radiation persists to influence cell growth, clonogenic assays were performed. The number of colonies were significantly increased in LNCaP and C4-2 cells treated with Zn and cisplatin (median 104.5, range 102-108 and median 38.5, range 30-45; p value = 0.03, Fig. 2F1-F2) and by 2-fold in Zn and radiation group compared to cisplatin or radiation treated cells without Zn (LNCaP cells; 99, 56; C4-2 cells; 98, 48, Fig. 2G1-G2).

DISCUSSION

The current study has established an excellent *in vitro* cell line model to study the effect of MT expression in PC. The results from our microarray analysis confirm that zinc treatment induces 0.33% of the 12,144 genes studied, the predominant of which are the MT. Others have studied the effect of induction of MT using zinc chloride on resistance to cisplatin in mice ¹¹. While such studies helped to establish the role of MT in resistance to chemotherapy, use of compounds like Hg at the study concentrations are usually toxic to humans and may not be translatable to human use. Because our experimental system uses high Zn concentration similar to human prostate, the results may be more readily translated to clinical settings. Because prostate cancer is a multifocal disease, high concentrations of Zn in normal appearing prostate glands surrounding prostate cancer may influence the phenotypic behavior of neighboring cancer glands. While our experiments simulate *in vivo* conditions of high zinc concentration, the caveat is that Zn concentrations within the heterogenous population of cells in prostate is variable and therefore our *in vitro* experiments may not truly replicate the prostate microenvironment.

Our study demonstrates a significant association between MT induction and resistance to cisplatin in PC cells. Others have demonstrated presence of a metallothionein-like zinc-binding protein in prostate cancer cell lines exhibiting relative resistance to cisplatin and nuclear localization of MT has been shown to be associated with cisplatin resistance in prostate cancer cell lines ^{12,13}. While further work is necessary to understand the mechanistic basis of MT induced resistance to cisplatin chemotherapy, it is conceivable that MT may function as an effective scavenger of trace metals including platinum, which may render cisplatin ineffective in PC. Although no specific MT inhibitor has yet been described, inhibition of cysteine synthesis by

propargylglycine has been shown to significantly reduce MT induction in mice inoculated with human or murine bladder tumor cells, which markedly enhanced the antitumor activity of cisplatin and other drugs ¹⁴. Because MT may be therapeutically manipulated, further studies are needed to explore MT as a biomarker for chemosensitivity of PC to cisplatin treatment and possibly improving the efficacy of cisplatin as radiosensitizer.

Our study also demonstrates that MT induction is associated with significant resistance to radiation treatment in PC. Radiation treatment fails in a minority of PC patients. Disease recurrence following radiation has been shown to be reduced by the use of neoadjuvant and adjuvant therapy in combination with radiation ¹⁵. Radiation causes injury to cells by inducing double strand breaks in DNA through free-radical production ¹⁶. Understanding the role of MT in resistance to radiation may provide novel strategies to improve radiation sensitivity. We have previously shown that MT 2A, the predominant MT isoform expressed in prostate, interacts with protein kinase D1 (PKD1) *in vitro* and *in vivo* ¹⁷. Activity of PKD1 can be altered by drugs such as Bryostatins-1, which may influence the function of MT. Alternatively small molecular inhibitors can also be developed to influence the activity of MT in PC. Because the prostate gland in human is readily accessible to imaging and therapeutic intervention such as direct needle injections, drugs altering MT activity may be administered directly into the prostate as a part of preparation for radiation treatment.

Our current study is limited to experiments performed using well-established PC cell lines. Further *in vivo* studies are needed to validate the findings. While our data suggests a strong and significant association between MT induction and resistance to radiation and cisplatin treatment,

further studies are needed to establish a direct causal relationship. Because resistance to cisplatin and radiation treatment can be due to a multitude of factors, altering the activity of MT in PC cells may not result in reversal of resistance to treatment in patients with PC. Nevertheless, our study has established a useful model of MT induction by high concentrations of zinc similar to human prostate gland and provides strong evidence supporting the potential role of MT in PC cells.

CONCLUSION

We have established an excellent *in vitro* cell line model system to study MT in PC. Our study suggests that MT induction by Zn is associated with resistance to cisplatin and radiation treatment in PC cells. Therapeutic targeting of MT may therefore provide a means to overcome cisplatin and radiation resistance in PC, although variable concentrations Zn within the heterogeneous population of prostate cells may pose additional therapeutic challenges.

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Figure Legends

Fig. 1A: C4-2 PC cells characteristically tolerate treatment with high concentrations of ZnSO₄. C4-2 PC cells and CP70 ovarian cancer cells (control) were grown to 80% in the presence of ZnSO₄. Cells were then incubated at 37°C for 72hours. Cell growth and viability was measured by MTS assay using the aqueous non-radioactive cell proliferation assay kit (Promega, Madison, WI). Whereas the C4-2 PC cells did not demonstrate significant decrease in viability in spite of high concentrations of ZnSO₄ up to 200μM, a majority of CP 70 ovarian cancer cells died at concentrations above 50μM.

Figs. 1B and 1C: Metallothionein expression is up regulated by ZnSO₄, but not by MgSO₄. LNCaP and C4-2 cells were plated out in 6 well plates 24hours prior to the addition of increasing concentrations of ZnSO₄, or MgSO₄. After 72hours of incubation the cells were lysed and analyzed by western blotting as described in method section. In fig 1B, lanes 1-4 show LNCaP cells, lanes 5-8 show C4-2 cells, treated with 0, 50, 100 and 150μM ZnSO₄. The positions of the protein markers are shown on the left. Protein kinase D1 is visible as a band in all lanes of 120kDa in size and its expression is not affected by zinc treatment, whereas, MT expression is clearly increased in a dose responsive manner. Induction of metallothionein expression in response to increasing ZnSO₄ concentration is shown by the increase in size of a band at around 10kDa. Fig 1C shows induction of MT expression in LNCaP and C4-2 cells treated with ZnSO₄ and no induction of MT in MgSO₄.treated cells.

Figs. 1D and 1E: Alterations in gene expression in LNCaP and C4-2 cells by 150 μ M Zinc Sulfate. Scatter plots showing the gene expression ratio changes in the cell lines LNCaP (D) and C4-2 (E) in the presence or absence of 150 μ M Zinc Sulfate. Dots above the upper dashed line indicate genes expressed significantly (>2 logs) higher in cells treated with Zn. The MT isoforms are visible as the most highly up regulated genes at the top of each plot. Dots within the dashed lines represent genes whose expression does not change significantly (<2 logs). Dots below the lower dashed line indicate genes expressed significantly (>2 logs) lower in cells treated with zinc. R^2 values indicate the linear regression of the data points.

Figs. 2A and 2B: Cisplatin dose response experiments. Experiments carried out at cisplatin doses ranging from 0 through 50 μ M, with cell viability measured at 3 days. LNCaP (Fig. 2A) and C4-2 (Fig. 2B) cells. Each experiment was carried out in triplicate. Cell growth and viability were measured by MTS assay, absorbance readings were taken at wavelengths of 490 and 655nm and the average absorbance for each cell line and concentration of drug was calculated. Error bars indicate mean standard error. The data points for zinc treated cells are shown as squares (■) with untreated cells shown as diamonds (◆).

Fig. 2C: Radiation sensitivity of LNCaP and C4-2 cells in the presence of 150 μ M ZnSO₄. LNCaP and C4-2 cells were grown to 80% confluence, plated in 96-well plates and incubated overnight in the presence or absence of 150 μ M ZnSO₄ as described in the previous experiment. Cells were then exposed to 5Gy of γ -radiation, grown for a further 72 hours and cell growth and viability was measured by MTS assay. The experiments were performed in triplicate and means

compared. Results are shown as a bar graph comparing cells exposed to radiation in the presence or absence of 150 μ M ZnSO₄. Error bars show standard error.

Fig. 2D-E: Analysis of apoptosis by flow cytometry: C4-2 cells treated with 10 μ M cisplatin or 5Gy radiation in presence or absence of 150 μ M of ZnSO₄ for 48 hours were analyzed. The figure demonstrates a significant decrease in apoptosis (sub G0 peak shown in box) in cells treated with ZnSO₄ compared to untreated cells; C4-2 cells only 8.73%, C4-2 cells + cisplatin: 14.24%, C4-2 cells +cisplatin +Zn: 9.12%; p value < 0.001(D1-D3), C4-2 cells: 0.34%, C4-2 cells+radiation: 6.35%,C4-2 cells+radiation+Zn: 1.13%; p value < 0.001(E1-E3).

Fig. 2F-G: A representative view of clonogenic assay demonstrating increased number of colonies in C4-2 cells treated with 10 μ M cisplatin (F1-2) or 5Gy radiation (G1-2) and 150 μ M of ZnSO₄ compared to cells not treated with ZnSO₄.

Figure 1

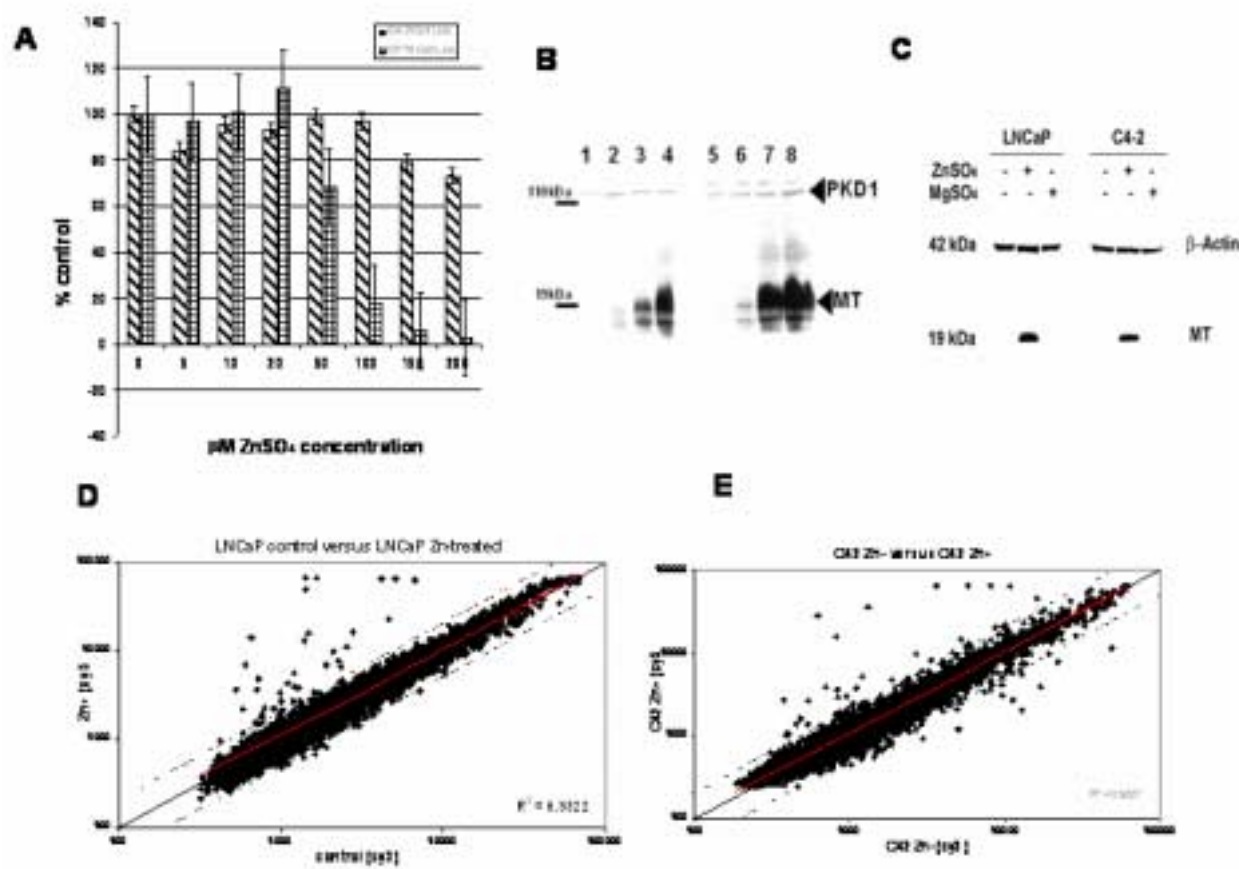


Figure 2

